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ARTICLE

Purification and Characterization of an Extracellular Lipase from the Fungus *Rhizopus delemar* [1]

The complete purification and characterization of an extracellular lipase (acylglycerol acylhydrolase, EC 3.1.1.3) from *R. delemar* is described. The final product was homogeneous as judged by electrophoresis in denaturing polyacrylamide gels and by isoelectric focusing, and was shown by means of an activity stain to be lipolytic. The purified enzyme had a monomer molecular weight of 30,300, an isoelectric point of 8.6, and approximately one monosaccharide moiety per molecule. *N*-Terminal sequence data (28 residues) and the amino acid composition of the lipase indicated that it corresponds to the product of a lipase-encoding cDNA previously isolated from *R. delemar*. Optimal activity occurred between pH 8.0 and 8.5. The activity and stability of the enzyme were maximum at 30°C. Divalent cations were required for activity, with barium, calcium and manganese conferring maximum activity. Activation by calcium was maximal at and above 10 mM. The lipase was not inactivated by reducing agents, sodium fluoride or phenylmethylsulfonyl fluoride. It was resistant to *N*-ethylmaleimide, and inactivated by *p*-chloromercuribenzoic acid in a manner which was not reversed by cysteine.

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In biological systems, lipases (acylglycerol acylhydrolases, EC 3.1.1.3) initiate the catabolism of fats and oils by hydrolyzing the fatty acyl ester bonds of acylglycerols. Work from several laboratories has established that the activities of lipases are not limited to this reaction. They also catalyze the synthesis and transesterification of glyceride (1–4) and phosphoglyceride (5,6) ester bonds, and the synthesis and hydrolysis of a variety of non-glyceride esters (7–12). In addition, lipases are active in both aqueous and non-aqueous solvent systems (1,4,10,11,13,14). It has therefore become evident that lipases have considerable biotechnological potential for the general synthesis and hydrolysis of esters (13–17).

Lipases differ from one another in their physical properties and biochemical features, such as substrate specificities, optimal reaction conditions, requirements for activators, and sensitivity to inhibitors (18,19). The mycelial fungus *Rhizopus delemar* produces at least three extracellular lipases (20, 21) which exhibit very high selectivity for the hydrolysis of primary, but not secondary, esters (22). Partially purified mixtures of these enzymes have been employed in a variety of reactions including ester (23) and glyceride synthesis (4,24,25), glyceride restructuring (2,26), and the exchange of the acyl groups of phospholipids (6).

The development of new uses for lipases as applied catalysts would be fostered by their purification and characterization. We report here the purification of one of the extracellular lipases of *R. delemar*. This enzyme corresponds to the product of a lipase-encoding (*LIP*) cDNA recently isolated from this organism (27).

MATERIALS AND METHODS

Enzyme production. Rhizopus delemar, ATCC 34612, was obtained from the American Type Culture Collection (Rockville, MD). The progeny of single spores were used as inocula for enzyme production. Growth medium contained 0.5% Casamino Acids (Difco, Detroit, MI), 30 mM glycerol, trace elements (28) and 5 μg biotin/L in a basal salts medium (29). Media were inoculated with 0.1% (vol/vol) of spore suspension (10^6 spores/L) and shaken at 150 rpm for 36 h at 30°C. The mycelia were then removed by filtration through cheesecloth, and the filtrates were stored at −20°C.

Enzyme purification. Culture filtrate was thawed in two and three liter batches and filtered through Whatman #1 paper. Sodium azide was added to a final concentration of 0.03% (wt/vol). Protease inhibitors were added to the final following concentrations: Phenylmethylsulfonyl fluoride (PMSF, 0.2 mM), pepstatin (1 μM), leupeptin (1 μM) and ethylenediaminetetraacetic acid disodium dihydrate (EDTA, 1 mM). The solution was applied at room temperature to an oleic acid affinity chromatography column (prepared as described below) with a bed volume of 20 mL at a cross sectional flow rate of 0.4 cm/min. Effluent was collected at intervals during loading and checked for lipase activity. When lipase adsorption fell below 40% of input, the column was chilled to 4°C and washed at a rate of 1 mL/min with: (i) 100 mL of 0.86 M NaCl in Buffer 1 (10 mM sodium phosphate, pH 6.0, containing the same inhibitors as were added to crude enzyme), (ii) 100 mL of Buffer 1, and (iii) a linear gradient of Triton X-100 from 0 to 0.5% in 320 mL of 10 mM sodium phosphate, pH 6.0. Fifteen-mL fractions were collected during the gradient.

Lipase-containing fractions from the affinity chromatography step were pooled and applied at 4°C to a CM-Sephadex C25 column (2.6 × 51.5 cm, Pharmacia LKB, Piscataway, NJ) equilibrated in Buffer 2 (10 mM sodium phosphate, pH 6.0, 1 mM EDTA). The column was washed with 400 mL of Buffer 2, followed by a linear gradient of NaCl from 0 to 0.5 M in 1 L of Buffer 2. The flow rate was 1 mL/min throughout. Fifteen-mL fractions were collected during the NaCl gradient.

A rapid and sensitive assay, wherein the hydrolysis of olive oil by lipase induces a change in the fluorescence of rhodamine B (Sigma, St. Louis, MO), was used to track lipase during purification (30). Two- to 100-μL aliquots of

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[1] Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Abbreviations: BME, β-mercaptoethanol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid disodium dihydrate; IEF, isoelectric focusing; kDa, kilodaltons; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid; pI, isoelectric point; PMSF, phenylmethylsulfonyl fluoride; PVP, polyvinylpyrrolidone; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; U, units of lipase activity; UV, ultraviolet.

potentially lipolytic samples were applied to an agar-solidified emulsion of olive oil and rhodamine B in a Petri dish. The dish was then incubated at room temperature for 5 to 60 min, whereupon active fractions could be detected by their bright fluorescence under ultraviolet (UV) light.

Synthesis of affinity chromatography resin. Oleic acid (1 mol free acid, Sigma), Affi-Gel 102 (20 mL, Bio-Rad Laboratories, Richmond, CA), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (10 mmol, Bio-Rad) were gently mixed overnight at room temperature in 28 mL of 70% aqueous dimethylformamide. The pH was held at 4.2 by the addition of 1N HCl with a constant titrating pH meter. The resin was washed in a column with 700 mL 80% ethanol, followed by two series of (a) 400 mL 0.1 N NH_4OH, and (b) 400 mL 80% ethanol. These were followed by 400 mL 0.1N NH_4OH, and 400 mL 80% methanol. Finally, the column was washed with 1 L 10 mM sodium phosphate, pH 6.0 containing 0.03% sodium azide. The continuous-service life of this resin exceeded two years.

Electrophoretic methods. 0.1% SDS-12% PAGE was performed according to Laemmli (31). The molecular weight of lipase was determined by reference to the mobilities of proteins of known molecular mass (Dalton Mk VII-L, Sigma).

Electrofocusing was performed on a PhastSystem (Pharmacia LKB) according to the manufacturers' directions using IEF 3-9 PhastGels (Pharmacia LKB) pre-equilibrated in a solution of 4M urea, 0.5% Nonidet P-40, and 4% pH 8-10.5 Pharmalytes (Pharmacia LKB). The isoelectric point (pI) of lipase was estimated by comparison of its mobility to those of standard proteins (High pI Calibration Kit, Pharmacia LKB) included on each gel.

Proteins were detected in gels by silver staining (32). Isoelectric focusing (IEF) gels were incubated 5 min in 20% trichloroacetic acid before staining, to eliminate interference by ampholytes.

Lipase activity was detected following IEF by placing the gel onto a solid lipase detection medium (30) containing olive oil and rohdamine B, incubating at room temperature for 100 min, and examining the medium under UV light for bright pink bands.

Determination of lipase activity. Lipolytic activity was quantitated by a continuous titrimetric assay using emulsified olive oil as substrate. A maximum of 0.58 mL of enzyme was added to 5 mL of pre-neutralized emulsion containing 18% (wt/vol) olive oil, 4.2% (wt/vol) gum arabic and 15 mM $CaCl_2$. A titrating pH meter (Radiometer, Copenhagen, Denmark) was employed to maintain the pH at a preset value by the addition of 0.1 N NaOH. Unless stated otherwise, incubations were carried out at 26°C with a set point pH of 7.5. Enzyme activity was calculated from the maximal rate of base addition, assuming molar equivalence between fatty acid release and base consumption. A unit of activity was defined as the release of one μmole of fatty acid per min under these conditions.

Purified lipase was dialyzed against deionized water prior to characterization. In determinations of the pH dependence of activity, the set point pH of the pH meter was adjusted to the values of interest. A thermostatted, water-jacketed reaction chamber was employed in determining the temperature dependence of activity. The effect of storage temperature on activity was measured by preincubating the enzyme at the desired temperature and assaying residual activity at 26°C. For investigations of the effect of cations upon lipase activity, 0.5% (wt/vol) polyvinylpyrrolidone [average mol. wt.: 360 kilodaltons (kDa), Sigma] replaced gum arabic as the emulsifier, and $CaCl_2$ was omitted from the assay mixture.

The calcium content of the gum arabic used as a substrate emulsifier was determined on a Perkin-Elmer (Norwalk, CT) PE 5000 Atomic Absorption Spectrophotometer with reference to $CaCl_2$ solutions of known concentrations.

Protein determination. Protein concentrations were determined according to Bradford (33) using the Bio-Rad Protein Assay Kit. For samples containing Triton X-100, the bicinchoninic acid assay (34) (Pierce, Rockford, IL) was employed. Bovine-γ-globulin was used as the standard in both assays.

Sugar content determination. The phenol-sulfuric acid method (35) was used to determine the degree of glycosylation of the purified lipase, using mannose as the reference standard. As another means of assessing the degree of glycosylation, individual samples of lipase were treated with endoglycosidase F and with *N*-glycosidase F (both from Boehringer Mannheim, Indianapolis, IN) (36), and examined for an alteration in electrophoretic mobility, relative to untreated lipase, by SDS-PAGE.

Amino acid analysis. Pure lipase was incubated in 5.7 N HCl for 24 h at 110°C. The amino acid compositions of the resulting hydrolysates were determined with a Beckman (Palo Alto, CA) Model 119CL amino-acid analyzer according to the manufacturers' instructions.

Amino acid sequence determination. *N*-Terminal sequence analysis was performed by automated Edman degradation using instrumentation, reagents and protocols from Applied Biosystems (Foster City, CA). A Model 470A gas-phase protein sequencer equipped with an online 120A PTH-analyzer and a Model 900A Control/Data Analysis Module were employed.

RESULTS AND DISCUSSION

Production and purification of lipase. We have produced and have purified to homogeneity an extracellular lipase from *R. delemar.* Glycerol was the main carbon source for the growth of the organism. In the majority of published papers describing lipase production, triglycerides have been the carbon source. However, there are disadvantages to their use, including media turbidity (which complicates measurement of the growth of the culture by optical methods), the insolubility of the fatty acids released by enzymatic hydrolysis, and the difficulty of removing unhydrolyzed triglyceride from solution prior to enzyme purification. We have found glycerol to be an acceptable carbon source for lipase production, resulting in enzyme levels comparable to those obtained with triglycerides, without the difficulties noted above.

Lipase eluted as a single peak from the oleic acid resin, during the Triton X-100 gradient, at a Triton concentration of approximately 0.25%. The output of two or three affinity chromatography run was routinely combined and taken to the CM-Sephadex step. Lipase eluted from this cation exchange column as a single sharp symmetrical peak at 0.25 M NaCl, the approximate mid-point of the gradient. The results of a typical purification are sum-

TABLE 1

Purification of *Rhizopus delemar* Extracellular Lipase

Step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification (-fold)
Filtered culture (22.8L)	350	259,208	741	1
Oleic acid affinity chromatography	19.7	97,926	4971	6.7
CM-Sephadex chromatography	10.1	77,145	7638	10.3

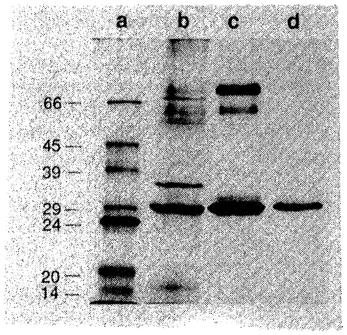

FIG. 1. SDS-PAGE of *R. delemar* lipase at various stages of purification. Proteins were detected by silver staining. Lane a, 2.25 μg of molecular mass marker preparation; lane b, 37.5 μg of culture filtrate; lane c, 3 μg of pooled lipase-positive fractions eluted from oleic acid affinity chromatography column; lane d, 1 μg of pooled lipolytic material obtained by CM-Sephadex column chromatography. The masses of the molecular weight marker proteins (kDa) are indicated in the left margin.

marized in Table 1. A 10.3-fold increase in specific activity was achieved, with a 30% recovery of activity. Purified preparations typically had specific activities of approximately 8000 units of lipase activity (U)/mg.

Enzyme characterization. As judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) (Fig. 1) and IEF (Fig. 2A), the final lipase preparation was homogeneous. When an IEF gel was incubated on lipase indicator media, a single fluorescent band was produced (Fig. 2B). The position of this band corresponded to that of the single protein band seen on IEF gels (Fig. 2A). Therefore, the purified protein is a lipase.

The molecular weight of the lipase, calculated by SDS-PAGE, was 30.3 kDa (Fig. 1). The isoelectric point was 8.6 (Fig. 2).

Amino acid composition and N*-terminal sequence.* The amino acid composition of the purified lipase (Table 2), indicates a minimum molecular weight of 29,762 (Trp was not determined). This agrees with the value determined by SDS-PAGE (above). As reported for other lipases, the protein is not enriched in hydrophobic residues, despite the fact that its natural substrates are very hydrophobic.

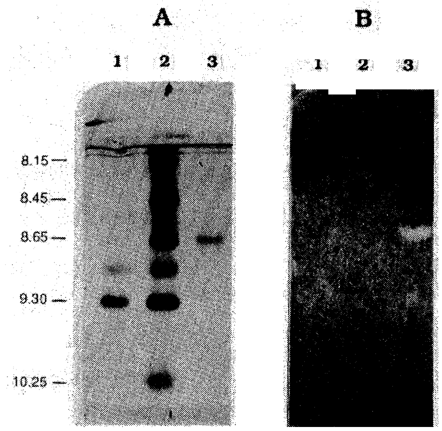

FIG. 2. Isoelectric focusing (IEF) of the purified lipase. (A) Silver stained IEF gel. Lane 1, 0.1 μg of trypsinogen standard; lane 2, 0.433 μg of IEF standard protein preparation; lane 3, 98 ng of pure lipase. The pI values of the standard proteins are indicated in the left margin. (B) Activity stain for lipase. An IEF gel identical to the one in Panel A was inverted onto lipase indicator media containing olive oil and rhodamine B, incubated for 100 min at room temperature, and photographed under UV light. The bright band indicates the site of lipase activity.

TABLE 2

Amino Acid Composition of the *R. delemar* Lipase[a]

Amino acid	Determined[b]	Predicted[c]
Ala	19	15
Arg	10	9
Asn	} 28	10
Asp		13
Cys	8	6
Gln	} 24	13
Glu		9
Gly	21	21
His	8	7
Ile	14	17
Leu	17	16
Lys	18	15
Met	2	1
Phe	14	15
Pro	16	15
Ser	22	24
Thr	21	22
Trp	n.d.[d]	3
Tyr	11	12
Val	19	26

[a] Expressed as number of amino acid residues per lipase molecule.

[b] Determined on the purified lipase as described in the text.

[c] Predicted by the nucleotide sequence of the *LIP* cDNA for amino acids +1→ +269 (27).

[d] n.d., Not determined.

Two nmol of purified lipase were subjected to *N*-terminal sequence determination. A low background was obtained throughout this operation, indicating that the preparation was very homogeneous. The *N*-terminus was not blocked. The sequence we determined is shown in Scheme 1. An identical sequence is encoded within the *R. delemar* lipase cDNA (27). Based on the gene sequence, the predicted molecular mass for the mature protein with this *N*-terminus is 29,592. This agrees with the value cal-

1 5 10
H_2N-Ser-Asp-Gly-Gly-Lys-Val-Val-Ala-Ala-Thr-Thr-Ala-Gln-

15 20 25
Ile-Gln-Glu-Phe-Thr-Lys-Tyr-Ala-Gly-Ile-Ala-Ala-Thr-Ala-Tyr-

SCHEME 1

culated for the pure lipase from SDS-PAGE data (above), and with that predicted from the amino acid composition of the enzyme (Table 2). In addition, the amino acid composition of the purified lipase agrees with that predicted from the cDNA sequence (27). We conclude that the lipase described here corresponds to the product of the cloned *LIP* cDNA and that it is initially synthesized as a larger polypeptide which is proteolytically cleaved to produce mature lipase.

The predicted pI of the mature lipase, calculated from the *LIP* cDNA, is 8.01 (27). The difference between this value and the pI determined here for the isolated fungal protein (8.6) may be related to the inclusion of urea and NP-40 in the IEF gel. These additives were absolutely required to detect lipase protein on the gels.

Pure lipase was determined to contain 0.53% carbohydrate. This corresponds to 0.88 monosaccharide residues per molecule. In agreement with this observation, neither endoglycosidase F nor *N*-glycosidase F treatment caused any alteration in the SDS-PAGE mobility of the lipase. In addition, the molecular weight of lipase, determined by SDS-PAGE of either crude (Haas, M.J., unpublished) or purified enzyme (above), is virtually identical to those calculated from the amino acid composition of the enzyme (above) and predicted from the sequence of the cloned lipase gene (27). All evidence therefore indicates that the lipase isolated here lacks substantial glycosylation. By contrast, a glycosylation level of 5–10% appears to be more typical of fungal lipases (37,38). Since the oleic acid affinity chromatography column was run at a low flow rate, this step of the purification required several weeks. It is possible that the lipase was initially glycosylated but that its carbohydrate moieties were removed during this operation by glycosidases endogenous to the crude enzyme preparation.

Properties of the lipase. The pH dependence of the activity of the pure enzyme is shown in Figure 3. Optimal activity occurred at slightly alkaline pH, with peak activity between pH 8.0 and 8.5. The enzyme exhibited 80% or more of maximal activity between pH 7.3 and 9.1. The fact that the lipase is maximally active near its pI may indicate that the binding of this enzyme to its hydrophobic substrate is best when its net charge is zero.

Lipase activity is temperature sensitive (Fig. 4). Maximum activity occurred at 30°C, with 80% of this value exhibited between 25 and 38°C. At and above 45°C, the activity fell to 0 within the first two min of incubation.

The thermal stability (15 min) of the lipase is illustrated in Figure 5. The enzyme was unstable above 30°C. Approximately 50% of the activity was lost during a 15 min preincubation at 42°C. Above 50°C, the enzyme was completely inactivated during a 5-min preincubation.

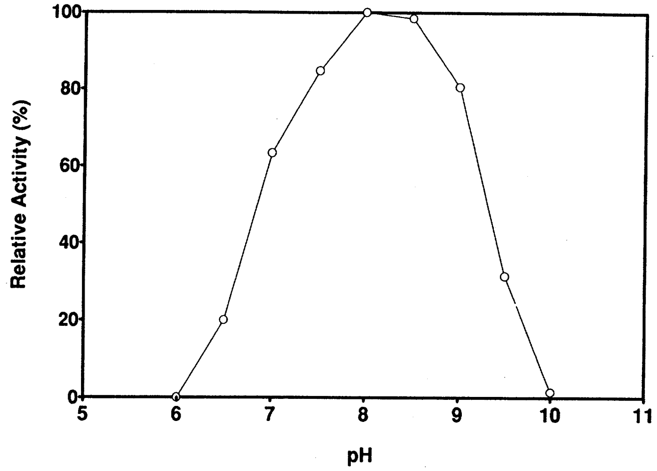

FIG. 3. The effect of pH on the activity of the pure lipase. The activity was assayed titrimetrically at various pH values as described in Materials and Methods, using olive oil as the substrate and gum arabic as emulsifier. Activities are expressed relative to that of the most active sample.

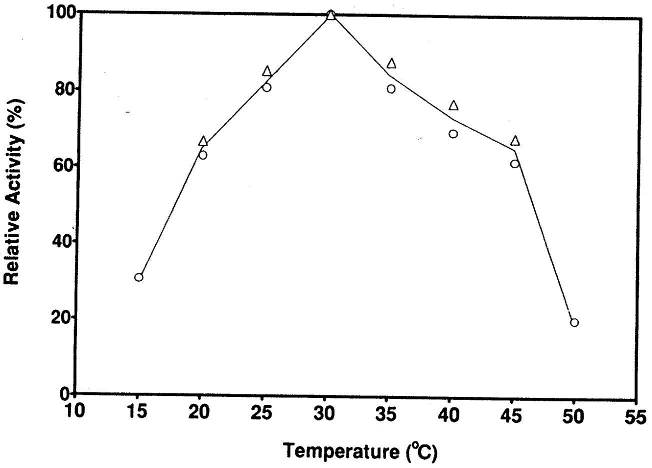

FIG. 4. Dependence of lipase activity upon temperature. Activity was assayed titrimetrically in a thermostatted, water-jacketed reaction vessel as described in Materials and Methods, using olive oil as the substrate and gum arabic as the emulsifier. The results of duplicate determinations are shown. Activities are expressed relative to that of the most active sample.

Some lipolytic enzymes require calcium for activity (18). It has been postulated that this stimulation is mediated through electrostatic interactions which mask repulsions either between the enzyme and its emulsified substrate or between the enzyme and product free fatty acids. To measure the effects of calcium and other ions, lipase activity was assayed in substrate emulsions where gum arabic, which contains inorganic ions, was replaced by the ion-free emulsifier PVP. The effects of the chloride salts of various cations, at concentrations of 10 mM, upon lipase activity were examined. With no additions, lipase activity was undetectable. Barium, calcium and man-

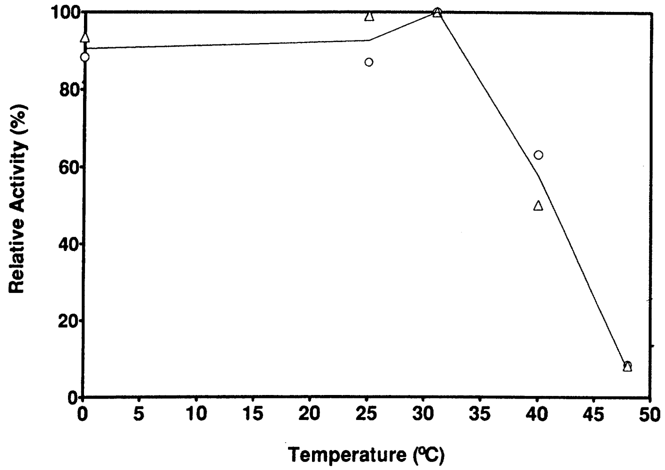

FIG. 5. Thermal stability of the lipase. Aliquots of enzyme were incubated at desired temperatures for 15 min. Lipolytic activity was then assayed titrimetrically at 26°C as described in Materials and Methods. The results of duplicate determinations are shown. Activities are expressed relative to that of the most active sample.

TABLE 3

Stability of *R. delemar* Lipase[a]

Treatment	Remaining activity (% of control)
BME, 10 or 25 mM, 64 h, 26°C	94 ± 4
DTT, 10 mM, 0.5 h, 26°C	112 ± 16
NaF, 10 mM, 0.5 h, 26°C	109 ± 2
PMSF, 0.2 mM, 0.5 h, 26°C	105 ± 1
NEM, 10 mM, 4 h, 26°C	101 ± 6
pCMB, 1 mM, 0.5 h, 26°C	87 ± 7
pCMB, 2 mM, 0.5 h, 26°C	5.1 ± 0.9
pCMB, 2 mM, 0.5 h, 26°C + cysteine, 5 mM, 0.66 h, 26°C	51 ± 6

[a]The activities are expressed as the mean and standard deviation of two determinations.

ganese (at 10 mM) substantially restored lipase activity (4400–5300 U/mg). Moderate amounts of activity were recovered in the presence of zinc, cobalt, copper and magnesium (700–3300 U/mg). No activity was detected upon the addition of sodium, potassium or ammonium salts.

The response to calcium was further investigated by testing its effects at several concentrations. Lipase activity rose steadily in PVP-emulsified systems as the calcium concentration increased, and was maximal above 10 mM. Gum arabic contains calcium. By atomic absorption spectroscopy, it was determined that the calcium concentration of gum arabic-emulsified assay mixtures was 68 mM. Nonetheless, the further addition of $CaCl_2$ stimulated the enzyme. Evidently, only a fraction of the calcium introduced by gum arabic is able to effect lipase activity. The stimulation by added $CaCl_2$ was maximal at and above 10 mM, resulting in an approximately three-fold increase in activity over that seen in the absence of added $CaCl_2$.

The effect of enzyme inhibitors upon lipase activity is shown in Table 3. The enzyme is resistant to the disulfide bond reducing agents dithiothreitol (DTT) and β-mercaptoethanol (BME). The predicted amino acid sequence of the *R. delemar* lipase is highly homologous to that of *Rhizomucor miehei*, which contains three disulfide bonds (27,42). The *R. delemar* lipase contains Cys at sites corresponding to the six Cys residues involved in disulfide formation in the *R. miehei* enzyme. It is reasonable to postulate that the *R. delemar* enzyme also has three disulfide bonds. The resistance of the *R. delemar* lipase to inactivation by reducing agents indicates that these disulfides are either inaccessible or are not essential for activity.

The enzyme was resistant to *N*-ethylmaleimide (NEM) (Table 3), consistent with the postulate that its sulfhydryls are involved in disulfide bonds. However, 2 mM *p*-chloromercuribenzoic acid (PCMB) did inhibit the lipase. Activity was only partially restored by cysteine (Table 3), suggesting that PCMB inhibition is due to action at some secondary site within the enzyme rather than to an interaction with a sulfhydryl group.

Sodium fluoride inhibits some lipases (39), while others are resistant to its effects (40). The *R. delemar* enzyme was not inhibited by fluoride (Table 3).

A serine-containing pentapeptide sequence has been postulated to form part of the active site of lipases, with the serine playing an active role in catalysis (41). The *R. delemar* lipase gene contains sequences encoding this conserved pentapeptide (27). However, the purified lipase was resistant to phenylmethylsulfonyl fluoride (PMSF), a potent serine-directed inhibitor (Table 3). Similar resistance has been reported for the *R. miehei* lipase (42), and attributed to a protective burying of the active site serine within the enzyme. Presumably this is also the case for the closely related *R. delemar* enzyme.

This is the first description of the complete purification and characterization of a *Rhizopus* lipase for which the cognate gene has also been isolated and analyzed. The size, pI and amino acid composition of the lipase reported here corresponds to those predicted for a peptide encoded by an internal portion of the cloned *LIP* cDNA (27). This correspondence led to the conclusion (27) that the lipase is initially synthesized as a precursor of molecular mass 42.1 kDa which undergoes proteolytic maturation to a smaller species with molecular mass of approximately 30 kDa.

The molecular mass of the *R. delemar* lipase is similar to that of a lipase from *Rhizomucor miehei* (38,42), and about 10 kDa smaller than that reported to date for a *Rhizopus* lipase. Iwai's group reported the partial purification of three extracellular lipases from *R. delemar* (20,21). The enzyme we have isolated is similar to their lipase C in terms of stability and pH and temperature requirements. However, the molecular mass of lipase C, determined by gel filtration, was 45 kDa, 15 kDa larger than the enzyme we have isolated. This larger molecular weight may be an overestimate of the true mass, since gel filtration was observed to overestimate the mass of another lipase, that from *R. miehei* (38). Alternatively, lipase C may have retained an associated glycopeptide during purification, as was demonstrated for *Rhizopus arrhizus* lipase (43–45). This lipase was isolated as a heterodimer consisting of a lipolytic fragment with a molecular mass of approximately 34 kDa, and a non-lipolytic glycopeptide of 8.5 kDa. The two fragments were separated by treatments such as acid precipitation, but this resulted in the inactivation of the lipase (45). Considering the close relationship between members of the genus *Rhizopus* (46), it seems probable that the *R. delemar* lipase is produced in a similar fashion, with the C lipase described by Iwai's group representing the heterodimer form of the enzyme we have isolated. The purification method here appears to allow the preparation of glycopeptide-free lipase which retains activity.

It is conceivable that the glycopeptides attached to the *R. arrhizus* lipases isolated by Laboureur and Labrousse (43) and Semeriva *et al.* (44,45) are lipase propeptides which remain associated with the enzyme after proteolytic maturation. Chiba *et al.* (47) reported the isolation of a lipase from a commercial preparation of crude *R. delemar* enzyme. The molecular weight of the enzyme was 41.3 kDa, similar to those of the enzymes studied by Laboureur and Labrousse (43) and Semeriva *et al.* (44,45). In size this enzyme corresponds to the prolipase encoded by the cloned *R. delemar* lipase gene (27). However, its amino acid composition (47) differs substantially from that predicted for the *R. delemar* prolipase (27). Therefore, it is unlikely that the glycopeptides found with some *Rhizopus* lipases are lipase propeptide fragments which have remained associated with the enzyme after proteolytic maturation of a precursor form of the enzyme.

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